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Glycyl-L-glutamine (Gly-L-Gln) is a dipeptide synthesized post-translationally from ß-endorphin. Gly-L-Gln is a major product of ß-endorphin processing in the brainstem, pituitary and several peripheral tissues, but little is known about its physiological function. The long term objective of this research is to characterize Gly-L-Gln's biological activities and test the feasibility of developing pharmacologic agents that mimic or antagonize its effects. This research has achieved four objectives: (1) Demonstrated that Gly-L-Gln is centrally active; it inhibits the hypotension and respiratory depression, but not the analgesia, produced by ß-endorphin. (2) Established the feasibility of developing peripherally active Gly-L-Gln analogs; specifically, we found that cyclo-Gly-L-Gln inhibits morphine and ß-endorphin-induced cardiorespiratory depression when injected peripherally. (3) Demonstrated that Gly-L-Gln produces trophic and neuroimmune effects in peripheral tissues. (4) Developed analytical methods for measuring Gly-L-Gln in biological tissues. (5) Generated initial evidence that saturable Gly-L-Gln binding sites are present in brain. These data support the hypothesis that Gly-L-Gln functions as a neurotransmitter in brain and a circulating hormone in the periphery.

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INTRODUCTION

Glycyl-L-glutamine is the C-terminal dipeptide fragment of the opioid peptide, ß-endorphin, (ß-endorphin-30-31) which is synthesized when ß-endorphin undergoes post-translational processing. ß-Endorphin processing has been intensely studied in recent years because it profoundly alters the peptide's analgetic activity, transforming ß-endorphin-1-31 from a highly potent opiate receptor agonist to an antagonist, ß-endorphin-1-27, and to opiate inactive forms, ß-endorphin-1-26 and the α-N-acetyl forms of all three peptides (Deakin et al., 1980; Nicolas and Li, 1985). Glycyl-L-glutamine, co-synthesized with ß-endorphin-1-27 when ß-endorphin-1-31 is endoproteolytically cleaved, has not been as thoroughly evaluated as the larger ß-endorphin forms, although existing evidence indicates that it, too, may participate in synaptic transmission. The objective of our research is to establish whether glycyl-L-glutamine acts as a neurotransmitter in brain and a circulating hormone in the periphery.

Evidence that glycyl-L-glutamine functions in synaptic transmission first arose from electrophysiologic studies by Parish et al. (1983) showing that iontophoretic glycyl-L-glutamine application inhibited the firing frequencies of brainstem neurons. This activity was not blocked by either naloxone or strychnine indicating that it was not mediated by either opioid or glycine receptors, respectively. Parish et al. (1985) also purified glycyl-L-glutamine from sheep brainstem and demonstrated that it is present in amounts equivalent to the sum of ß-endorphin-1-27 and ß-endorphin-1-26, as one would predict. Immunohistochemical studies have also demonstrated that glycyl-L-glutamine is localized in the intermediate, but not in the anterior lobe of the pituitary (Plishka et al., 1985) where ß-endorphin does not undergo C-terminal cleavage (Eipper and Mains, 1980; O'Donohue and Dorsa, 1982). Hence these studies indicate that glycyl-L-glutamine is present in brain and pituitary and that it inhibits the electrical activity of brainstem neurons.

Several additional lines of evidence further support the concept that glycyl-L-glutamine acts as a neurotransmitter. Behavioral studies revealed that glycyl-L-glutamine inhibits ß-endorphin induced grooming in rats, a response thought to reflect mechanisms of attention and arousal (Hirsch and O'Donohue, 1985). Glycyl-L-glutamine is also thought to function as a trophic agent at the neuromuscular junction (Lotwick et al., 1990) and in autonomic ganglia (Koelle et al., 1988). In both tissues, neuronal innervation induces synaptic acetylcholinesterase (AChE) activity; glycyl-L-glutamine produces a comparable effect, suggesting that it may be the neurotrophic agent mediating the response. ß-endorphin has the opposite effect, reducing AChE, and may be responsible for the subsequent lowering of AChE activity observed during synaptic reorganization (Haynes et al., 1984).

Glycyl-L-glutamine may also play a role in the neuroendocrine regulation of the immune system. A large body of evidence supports the concept that the central nervous system influences immune response and much of this work focuses on the role of POMC peptides as neuroimmune mediators (Morley et al., 1987). McCain et al. showed that low concentrations of glycyl-L-glutamine enhance phytohemagglutinin (PHA) induced T-lymphocyte proliferation (McCain et al., 1987). This was a key finding because the PHA response, which mimics antigen induced lymphocyte activation, is often used as a measure of immune competence. Once again, ß-endorphin had the opposite effect, suppressing PHA-induced proliferation (McCain et al., 1987). These observations suggest that glycyl-L-glutamine release from the intermediate pituitary may partially counteract stress-induced suppression of immune function.

These findings support the concept that glycyl-L-glutamine, like other ß-endorphin peptides, functions both as a neurotransmitter in brain and as a circulating hormone in the periphery; however, the basic studies required to firmly establish such a role for glycyl-L-glutamine have not been performed. Several criteria must be fulfilled. First, glycyl-L-glutamine's pharmacologic spectrum of activity must be definitively established, emphasizing its interactions with ß-endorphin and opiate drugs. Second, it must be demonstrated that glycyl-L-glutamine is present in ß-endorphin releasing neurons and endocrine cells. And third, receptors for glycyl-L-glutamine must be unequivocally identified and thoroughly characterized. We predict that glycyl-L-glutamine receptors exhibit 'synaptic specificity', meaning they are found only in ß-endorphin neuronal synapses. The concept of synaptic specificity is important because it means that drugs targeted on glycyl-L-glutamine receptors will act only at ß-endorphin neuronal synapses, unlike all existing opiate drugs which interact with different opioid receptor subtypes present in all three opioid peptide systems. Thus, the longer term objective of this research is to establish the data base necessary to design therapeutic agents targeted on glycyl-L-glutamine receptors to selectively modify the biological effects of both glycyl-L-glutamine and 3-endorphin.

RESULTS AND DISCUSSION

A. Pharmacology: CNS Effects

1. Central Cardiovascular Regulation (Appendix I): ß-Endorphin generates severe hypotension, bradycardia and respiratory depression when administered intracerebroventricularly (icv) to rats and other species (Petty and Sitsen 1989; Millington and Hirsch, 1994). These effects are thought to be mediated, in large part, by mu opioid receptors the nucleus of the solitary tract and adjacent brainstem nuclei. Collectively, these observations support the hypothesis that ß-endorphin plays an important role in cardiovascular homeostasis.

This conclusion must be tempered by the consideration that ß-endorphin is extensively processed in brainstem neurons; α-N-acetyl-ß-endorphin-(1-27), α-N-acetyl-ß-endorphin-(1-26) and ß-endorphin-(1-26) are the predominant forms (Zakarian and Smyth, 1982) Unlike ß-endorphin-(1-31), these N- and C-terminally modified ß-endorphin peptides display little or no affinity for opioid receptors and have no effect on peripheral hemodynamics when injected centrally (Hirsch and Millington, 1991). Glycyl-L-glutamine is thus a major end-product of ß-endorphin processing in the brainstem because it is produced in amounts equivalent to the combined concentrations of ß-endorphin-(1-27), ß-endorphin-(1-26), α-N-acetyl-ß-endorphin-(1-27) and α-N-acetyl-ß-endorphin-(1-26) (Parish et al., 1982). Consequently, brainstem glycyl-L-glutamine concentrations substantially exceed ß-endorphin levels. This raises the possibility that glycyl-L-glutamine may be an important product of ß-endorphin processing, not only quantitatively, but functionally as well. Despite its quantitative significance, glycyl-L-glutamine's physiological functions have not been thoroughly evaluated and its role in cardiovascular homeostasis, if any, is unknown.

a) Glycyl-L-glutamine Inhibits ß-Endorphin-Induced Hypotension

We found that glycyl-L-glutamine inhibited ß-endorphin-induced hypotension, but not bradycardia, when administered icv to pentobarbital anesthetized rats 15 min after ß-endorphin injection. ß-Endorphin (0.5 nmol) followed by saline injection produced a rapid and sustained reduction in arterial blood pressure, lowering MAP by 42.3 ± 6.2 mm Hg within 60 min. Glycyl-L-glutamine (0.3, 0.6, 1.0 or 10.0 nmol) dose-dependently inhibited ß-endorphin-elicited hypotension; the lowest significant inhibitory dose was 1.0 nmol. In contrast, glycyl-L-glutamine had no significant effect on ß-endorphin-induced bradycardia. ß-Endorphin-reduced heart rate by 154 ± 14 beats/min within 60 min from a baseline of 389 ± 15 beats/min. Glycyl-L-glutamine partially restored heart rate to control values but its effect was neither statistically significant nor dose-dependent. Glycyl-L-glutamine had no effect on MAP or heart rate when administered icv to animals that had not received ß-endorphin at doses as high as 100 nmol. N-Acetyl-glycyl-L-glutamine and glycyl-L-glutamate also reversed ß-endorphin-induced hypotension although they were less potent than glycyl-L-glutamine. Glycyl-D-glutamine was ineffective, suggesting that the dipeptide response is stereoselective.

One important caveat to these experiments, is the possibility that the observed effects resulted from the hydrolysis of glycyl-L-glutamine to its constituent amino acids. Indeed, icv glycine lowers MAP albeit at relatively high doses (1 umol or more) (Persson, 1980). We found that co-administration of 1 nmol of each amino acid, glycine and glutamine, had no effect whatsoever on ß-endorphin-induced hypotension. Consistent with published reports, a higher dose (1.0 µmol of each amino acid) potentiated, rather than inhibited, the hypotensive response to ß-endorphin. These findings indicate that glycyl-L-glutamine hydrolysis does not account for its cardiovascular effects and further suggest that

glycyl-L-glutamine does not act through glycine receptors.

b) Respiratory Depression: Like morphine, ß-endorphin produces respiratory depression when injected centrally (Shook et al., 1990). To determine if glycyl-L-glutamine inhibits the respiratory depressant effect of ß-endorphin, we measured blood gases immediately before and 45 min after ß-endorphin injection; as in previous experiments, glycyl-L-glutamine or saline was administered 15 min after ß-endorphin. As expected, ß-endorphin followed by saline injection increased pCO_2 and lowered pO_2 and pH although HCO_3^- and base excess concentrations did not change significantly.

Glycyl-L-glutamine attenuated the hypercapnia and hypoxia induced by ß-endorphin. Both the rise in plasma pCO_2 and fall in pO_2 elicited by ß-endorphin were significantly diminished by subsequent glycyl-L-glutamine injection; indeed, pCO_2 and pO_2 were not significantly different than baseline values following sequential ß-endorphin and glycyl-L-glutamine administration. Glycyl-L-glutamine did not influence the reduction in plasma pH caused by ß-endorphin, however. When administered icv to rats that had not been pretreated with ß-endorphin, glycyl-L-glutamine (1.0 nmol) had no effect on pCO_2; pO_2 or pH measured immediately before and 30 min post-injection. These data indicate that glycyl-L-glutamine attenuates ß-endorphin-induced hypercapnia and hypoxia but has no effect on blood gases when administered independently.

c) Glycyl-L-glutamine Does Not Displace ^{3}H-Naloxone Binding: The cardiorespiratory effects of ß-endorphin are thought to be mediated by mu opioid receptors (Petty and Sitsen, 1989; Millington and Hirsch, 1994) raising the possibility that glycyl-L-glutamine inhibits ß-endorphin's effects by acting as a mu receptor antagonist. To test this, we conducted receptor binding experiments using [^{3}H]naloxone as a ligand. [^{3}H]Naloxone binding to rat brain homogenates was saturable with K_d and B_{max} values of 1.5 nM and 138 fmol/mg protein, respectively, consistent with previous reports (Wood et al., 1981). Non-specific binding was less than 25% total binding. [^{3}H]Naloxone binding was displaced by morphine, ß-endorphin and ß-endorphin-(1-27) with K_i values in the nM range comparable to previously published data (Wood, et al., 1981).

Glycyl-L-glutamine failed to displace [^{3}H]naloxone binding at concentrations ranging from 1 pM to 10 mM (Table 4). For example, [^{3}H]naloxone binding in the presence of 10 mM glycyl-L-glutamine, the highest concentration tested, was essentially the same as control values (102.5 $\pm$ 6.7 % control). Glycyl-L-glutamine is therefore unlikely to inhibit ß-endorphin-induced hypotension by acting as an opioid receptor antagonist.

d) Cyclo-glycyl-L-glutamine: A Peripherally Active Analog: A longer term objective of this research is to test the feasibility of developing peripherally active glycyl-L-glutamine analogs to selectively inhibit the cardiorespiratory depression, but not the analgesia, produced by opiate drugs. Toward this end, we tested

whether cyclo-glycyl-L-glutamine, a cyclic analog formed by coupling glycyl-L-glutamine's N- and C-terminals, inhibited ß-endorphin-induced hypotension. Cyclo-glycyl-L-glutamine was of interest in light of evidence that other cyclic dipeptides are metabolically stable, and because they are non-polar, permeate the blood-brain barrier (Hoffman et al., 1977).

We found that cyclo-glycyl-L-glutamine effectively blocks ß-endorphin-induced hypotension. Initially, we tested whether cyclo-glycyl-L-glutamine was effective when injected centrally. We found that, like glycyl-L-glutamine, cyclo-glycyl-L-glutamine (0.3, 0.6 or 1.0 nmol) produced a dose-related inhibition of ß-endorphin-induced hypotension; indeed, it was nearly as potent as the linear form. In subsequent experiments, we found that intra-arterial (ia) cyclo-glycyl-L-glutamine injection (5 mg/kg) also inhibited the hypotension induced by central ß-endorphin injection. A comparable intra-arterial dose of glycyl-L-glutamine was ineffective suggesting that cyclo-glycyl-L-glutamine is unlikely to act at peripheral sites. Cyclo-glycyl-L-glutamine (1, 5 or 50 mg/kg; ia), injected alone, was similarly without effect on peripheral hemodynamics. These results indicate that cyclo-glycyl-L-glutamine effectively blocks the hypotensive effects of ß-endorphin when injected either icv or intra-arterially.

Our final objective, was to test whether glycyl-L-glutamine blocks the cardiorespiratory depression produced by morphine and other opiates. In our initial experiments, we treated pentobarbital anesthetized rats with a relatively high morphine dose (100 nmol) icv, followed 15 min thereafter, by glycyl-L-glutamine (10 nmol icv). Morphine alone produced profound hypotension, lowering MAP by up to 80 mm Hg; glycyl-L-glutamine completely blocked the response. Cyclo-glycyl-L-glutamine (5 mg/kg, ia) also effectively blocked morphine (100 nmol icv) induced hypotension. Furthermore, cyclo-glycyl-L-glutamine (5 mg/kg ia) inhibited the respiratory depression induced by central morphine (50 nmol icv) injection. Together, these data indicate that both glycyl-L-glutamine and cyclo-glycyl-L-glutamine inhibit the cardiorespiratory depression produced by, not only ß-endorphin, but opiate drugs as well.

The mechanism responsible for glycyl-L-glutamine's inhibitory effects on opioid-induced cardiorespiratory depression remains to be determined. However, the profound hypoxia and hypercapnia produced by both morphine and ß-endorphin suggests that the hypotensive response to opioids may be secondary to respiratory depression in anesthetized animals. In support of this conjecture, we found that morphine and ß-endorphin were substantially less potent hypotensive agents when administered to conscious or mechanically ventilated animals. This raises the intriguing possibility that glycyl-L-glutamine may effectively oppose opioid-induced respiratory depression, a significant side effect of opiate drugs. The prospect that glycyl-L-glutamine may inhibit one of the side effects of opiate analgetics prompted us to examine whether the dipeptide also blocks the analgetic effects of opioids.

2. Antinociception: To determine whether glycyl-L-glutamine modulates the antinociceptive effects of ß-endorphin we used the tail flick test which measures the response time for a rat to remove its tail from a beam of light. Initial experiments affirmed that the technique generated consistent baseline data by testing the antinociceptive effects of morphine (3.5 mg/kg ip; 30 or 100 nmol, icv) and ß-endorphin (1, 1.5 or 3 nmol; icv). Consistent with previous reports, ß-endorphin generated a prolonged increase in tail flick response latencies, which were maximal 40 min after injection and persisted for up to two hours.

After establishing consistent baseline data, we tested whether glycyl-L-glutamine modulates the antinociceptive response to ß-endorphin. Our initial results indicated that glycyl-L-glutamine inhibited the ß-endorphin effect when the two peptides were co-injected icv. Glycyl-L-glutamine (15 nmol) significantly reduced the maximal response to ß-endorphin (1.5 nmol) by 67%. A higher glycyl-L-glutamine dose (50 nmol) produced a small, but non-significant inhibitory effect (39%) on ß-endorphin-induced antinociception, but neither lower (5 nmol) nor higher (150 or 500 nmol) glycyl-L-glutamine doses had any effect. Glycyl-L-glutamine, administered alone (5 nmol - 1.5 µmol) was ineffective. Hence, our results thus far are somewhat equivocal; glycyl-L-glutamine appears to inhibit ß-endorphin-induced antinociception over a narrow dose range but produces no effect when administered alone. Future experiments will test whether glycyl-L-glutamine modulates the antinociceptive response to morphine. Collectively, these results indicate that glycyl-L-glutamine inhibits the cardiorespiratory depression generated by ß-endorphin, but has no consistent effect on ß-endorphin-induced antinociception in the same dose range.

3. Thermoregulation (Appendix II): The results of our studies on cardiovascular function suggests that glycyl-L-glutamine specifically modulates the effects of ß-endorphin, from which it is synthesized, as well as other opioids which activate the same receptors. Alternatively, it is possible that glycyl-L-glutamine functions as an inhibitory modulator of central effects produced by other peptides synthesized from the ß-endorphin prohormone, pro-opiomelanocortin (POMC). Fortuitously, one of our collaborators, Dr. Garth Resch, had recently demonstrated that α-MSH induces hyperthermia when injected into specific thermoregulatory sites the medial preoptic area (mPOA) of the hypothalamus (Resch and Simpson, 1991). This finding provided us with an ideal opportunity to test whether glycyl-L-glutamine modulates the central actions of a second POMC peptide, α-MSH.

We found that glycyl-L-glutamine completely blocked the hyperthermic response to α-MSH (Resch and Millington, 1993). α-MSH (60 pmol) injection into the mPOA generated a sustained elevation in colonic temperature (Tc), reaching a maximum of 0.85 ± 0.19 °C within 45 min. Co-administration of glycyl-L-glutamine (3 nmol) completely blocked the response to α-MSH, maintaining Tc at baseline levels throughout the 45 min test period. Glycyl-L-

glutamine, injected alone produced no significant effect on Tc, however. The inhibitory response to glycyl-L-glutamine did not result from hydrolysis of the peptide to its constituent amino acids because co-administration of equimolar amounts of glycine and glutamine had no effect whatsoever on α-MSH thermogenesis. Interestingly, the modulatory effect of glycyl-L-glutamine was of unexpectedly long duration. Pretreatment with glycyl-L-glutamine significantly attenuated the response to α-MSH given 3 h or 24 h thereafter although it was fully restored within 48 h. The attenuated response did not result from desensitization because control experiments showed that two α-MSH injections, given 4 h or 48 h apart, produced the same rise in Tc. Thus, glycyl-L-glutamine produces a long-lasting inhibition of the thermoregulatory response to α-MSH without directly altering thermoregulation when given alone. These aggregate results indicate that glycyl-L-glutamine modulates certain of the central effects produced by both ß-endorphin and α-MSH.

These results prompted us to consider whether, in addition to α-MSH, glycyl-L-glutamine inhibits the hyperthermia induced by other hyperthermic agents. The mPOA is an important site of action for interleukin 1ß (IL-1ß) and other pyrogens which are released from circulating lymphocytes as part of the acute phase response to infection. The pyrogenic effects of IL-1ß and related pyrogens are thought to be mediated by prostaglandin E_2 (PGE_2) (Kluger, 1991). Hence, our initial studies tested whether glycyl-L-glutamine inhibited the hyperthermic response to mPOA PGE_2 injection. We found that glycyl-L-glutamine (3 nmol) completely blocked the hyperthermia induced by PGE_2 (3 nmol) (unpublished data). In subsequent experiments, we tested whether IL-1ß-induced fever was similarly affected. IL-1ß (1 I.U.) alone, generated a prolonged rise in body temperature which lasted for the entire 2 h duration of the experiment. Co-injection of glycyl-L-glutamine (3 nmol) completely blocked the response. These results indicate that the thermomodulatory effects of glycyl-L-glutamine are not limited to POMC peptides. Moreover, they show that glycyl-L-glutamine blocks the hyperthermia induced by typical pyrogenic agents suggesting that it may serve an antipyretic thermoregulatory role.

B. Pharmacology: Peripheral Effects

1. Trophic Effects on Cardiac Myocytes: The first evidence that glycyl-L-glutamine may function as a hormone and/or neuromodulator in peripheral tissues was the finding that glycyl-L-glutamine induces the expression of acetylcholinesterase in sympathetic ganglia (Koelle et al., 1988) and skeletal muscle cells (Lotwick et al., 1990). These tissues do not normally synthesize POMC peptides, however, suggesting that glycyl-L-glutamine acts as a circulating hormone, following its release from the pituitary. Recent reports that ß-endorphin immunoreactivity is localized in cardiac tissue (Forman et al., 1989) prompted us to examine whether glycyl-L-glutamine produces similar trophic actions on AChE in

cardiac myocytes. Hence, our working hypothesis was that glycyl-L-glutamine may be synthesized and released from cardiac myocytes to regulate cardiac function locally.

AChE molecular forms can be divided into two classes, asymmetric or globular, based on the presence or absence of a collagen-like tail. The predominant asymmetric form, A_{12}, is localized in the basal lamina and is thought to be involved in the physiological regulation of acetylcholine hydrolysis (Rieger et al., 1980). Relatively high A_{12} AChE activity is detectable in intact rat heart, but not in cardiac myocytes in tissue culture suggesting that a trophic factor maintains A_{12} AChE expression *in vivo*. To determine whether glycyl-L-glutamine induces expression of A_{12} AChE, we prepared myocyte cultures by enzymatically dissociating ventricles from 2-4 day old rats; the myocytes were confluent and beating for at least 48 h prior to experimentation. AChE molecular forms were analyzed by sucrose density gradient fractionation followed by analysis of AChE activity in the resulting fractions.

Previous studies by our collaborator, Dr. Nyquist-Battie (1987), showed that A_{12} AChE is a relatively minor component of total AChE activity in adult rat heart, comprising approximately 5% of ventricular AChE activity. We found, in myocyte cultures, that a comparable proportion of total AChE was attributable to the A_{12} AChE form in myocyte prepared from pre-natal rat ventricles (8 $\pm$ 0.3 %). As in the adult, globular forms predominated, including monomeric, G_1 (58 $\pm$ 1 %), and tetrameric, G_4 (34 $\pm$ 3 %) (Nyquist-Battie et al., 1993). However, in post-natal myocyte cultures, A_{12} AChE was not detectable. Co-incubation with glycyl-L-glutamine (1 μM) for 72h increased the proportional amount of A_{12} AChE, restoring its activity to that of pre-natal cultures (8 $\pm$ 0.8 %). Corresponding decreases occurred in G_1 (53 $\pm$ 3 %) and G_4 (33 $\pm$ 3%) AChE. Glycyl-L-glutamine did not change the specific activity of either intracellular or secreted AChE. The response to glycyl-L-glutamine appeared to be relatively specific to the extent that neither glycyl-L-glutamate nor glycyl-D-glutamine had any effect on AchE expression. These results provide the first evidence that glycyl-L-glutamine produces trophic effects in the heart.

a) ß-Endorphin Processing in Rat Heart (Appendix III): The observation that glycyl-L-glutamine induces trophic effects in cardiac cells supports the hypothesis that glycyl-L-glutamine acts as an autocrine factor produced locally in heart. Recent evidence that ß-endorphin immunoreactivity are localized in rat heart suggests that glycyl-L-glutamine may be synthesized by cardiac cells (Forman et al., 1989). To obtain indirect evidence as to whether glycyl-L-glutamine may also be synthesized in cardiac tissue, we analyzed the molecular forms of ß-endorphin.

Sequential gel filtration and ion exchange HPLC analyses revealed that ß-endorphin-1-31 is extensively converted to endoproteolytic cleaved and N-acetylated derivatives in rat heart. Indeed, ß-endorphin-1-31 comprised only 16 $\pm$ 4 % of total ß-

endorphin immunoreactivity in heart extracts (Millington et al., 1993). The single major form of cardiac ß-endorphin immunoreactivity co-eluted with N-acetyl-ß-endorphin-1-31. In addition, ß-endorphin-1-27, ß-endorphin-1-26 and their N-acetylated con-geners each comprised between eight and sixteen percent of total ß-endorphin immunoreactivity. Hence, virtually all the ß-endorphin peptides synthesized in heart are inactive as opioid receptor agonists.

These studies further revealed that ß-lipotropin, ACTH and α-MSH were also localized in rat heart. Only low levels of ß-lipotropin and ACTH were detected, indicating that they primarily serve as precursors to ß-endorphin and α-MSH, respectively. Reverse phase HPLC analysis further revealed that multiple forms of α-MSH were also present, and like ß-endorphin, acetylated peptides, α-MSH and N,O, diacetyl-α-MSH, redominated. These studies are the first to show that multiple forms of α-MSH and ß-endorphin are localized in heart and provide strong inferential evidence that glycyl-L-glutamine is also expressed.

It remained to be determined, however, whether the POMC peptides found in rat heart are synthesized by cardiac myocytes or by autonomic neurons innervating the heart. To discern between these two possibilities, we initiated immunohistochemical experiments using antisera against α-MSH, ß-endorphin and, for comparative purposes, atrial natriuretic factor (ANF). This revealed that myocytes do, indeed, contain immunoreactive α-MSH and ß-endorphin (Nyquist-Battie et al., 1994). Virtually every myocyte was stained, although the staining intensity was substantially higher in atria than ventricles. In neonatal heart, staining was of equivalent intensity in both regions. ANF immunoreactivity was similarly distributed in both adult and neonatal heart, consistent with earlier reports (Ruskoaho, 1992). Axons were not stained by any of the antisera.

To further test the hypothesis that myocytes synthesize ß-endorphin, α-MSH and related POMC-derived peptides, we examined whether POMC mRNA is localized in rat heart by using *in situ* hybridization with colorimetric detection. We found that POMC mRNA is also detectable in rat atria; virtually all cells were labeled, albeit with varying intensities (Nyquist-Battie et al., 1994). Relatively intense staining was also present in cultures prepared from neonatal atrial or ventricular myocytes. These data provide preliminary evidence that POMC is synthesized and processed by cardiac myocytes. Experiments now in progress seek to confirm these results by Northern blot analysis of POMC mRNA in rat heart and myocyte cultures.

3. Neuroimmune Effects: In addition to acting on peripheral tissues, a wealth of evidence indicates that ß-endorphin peptides modulate immune function. For example, ß-endorphin enhances phytohemagglutinin (PHA) induced T-lymphocyte proliferation, a measure of the ability of lymphocytes to respond to antigenic stimuli which

is often used to test immune competence (Gilmore and Weiner, 1988) Little is known about the role of glycyl-L-glutamine, however.

To determine whether glycyl-L-glutamine is also involved in neuroimmune regulation, we examined the effect of ß-endorphin and glycyl-L-glutamine on lymphocyte proliferation in a human T-lymphocyte cell line, Jurkat E6-1. Mitogenic stimulation induces a rapid increase in c-myc mRNA levels, both in normal lymphocytes and in Jurkat E6-1 cells (Hough et al., 1990). We found that glycyl-L-glutamine (30 nM) produced a 2.5-fold increase in concanavalin A (50 ng/ml) stimulated c-myc mRNA expression when co-incubated with sub-effective amounts of ß-endorphin; when added to the cell cultures alone, glycyl-L-glutamine had no significant effect. Interestingly, these effects did not appear to be mediated by interleukin-2 (IL-2) because ß-endorphin had no effect on IL-2 release from Jurkat cells. While yet preliminary, these results suggest that glycyl-L-glutamine potentiates ß-endorphin induced c-myc expression.

a) ß-Endorphin Processing in the Human Pituitary Gland (Appendix IV): The finding that glycyl-L-glutamine modulates the effect of ß-endorphin on a human lymphocyte cell line raised the question as to the source of glycyl-L-glutamine which normally regulates immune cell function. One source may be the lymphocytes themselves, which are known to express ß-endorphin and other POMC derived peptides (Morley et al., 1987). Alternatively, glycyl-L-glutamine may be released from the intermediate pituitary lobe which, in the rat, produces large amounts of glycyl-L-glutamine (Plishka et al., 1985). To determine whether the human pituitary also synthesizes glycyl-L-glutamine, we analyzed the molecular forms of ß-endorphin, extending our previous studies of ß-endorphin processing in human brain (Millington and Smith, 1991). This revealed that only small amounts of C-terminally shortened ß-endorphin peptides are localized in the human pituitary (Evans et al., 1993). ß-Endorphin-(1-31) was the predominate ß-endorphin peptide, comprising 85% of total ß-endorphin immunoreactivity. This indicates that the human pituitary does not produce significant amounts of glycyl-L-glutamine; lymphocytes may be the source of the glycyl-L-glutamine which modulates immune function.

Correlative immunohistochemical studies further revealed two unexpected findings regarding pituitary ß-endorphin processing. We used several antisera to identified pituitary cells that synthesize ß-endorphin, including one which specifically recognizes N-acetyl-ß-endorphin peptides. As expected, this antiserum stained a small number of cells along the border between the anterior and neural lobes where the remnants of the fetal intermediate lobe are localized. Unexpectedly, however, N-acetyl-ß-endorphin, as well as α-MSH, immunoreactive cells were also dispersed throughout the anterior lobe. This indicates that cells resembling melanotrophs, biochemically, are distributed throughout the human pituitary, unlike virtually every other mammalian species in which N-acetyl-ß-endorphin and α-MSH are localized in the intermediate, but not the anterior lobe.

These studies also revealed that ß-endorphin immunoreactive axons were present within the human neural lobe (Manning et al., 1993). ACTH immunoreactivity has previously been reported in rat neural lobe axons (Knigge and Joseph, 1982) but innervation of the human neural lobe by POMC neurons has not been previously reported. We showed that human neural lobe axons were also labeled by antisera to ACTH and α-MSH, confirming their identity as POMC axons. Unexpectedly, a smaller number of axons were intensely stained by the N-acetyl-ß-endorphin antisera, suggesting that a subpopulation of neural lobe POMC axons N-acetylate ß-endorphin peptides.

C. Analytical Methods:

An additional broad objective of our research has been to develop analytical methods for isolating and measuring glycyl-L-glutamine concentrations in order to study its distribution in brain and release in vitro. We also plan to use radiolabeled glycyl-L-glutamine to investigate glycyl-L-glutamine binding sites and metabolism in brain, and for these applications, it is essential to develop methods for separating glycyl-L-glutamine from its constituent amino acids, glycine and glutamine. We have had good success in developing HPLC methods for separating and quantifying glycyl-L-glutamine although our efforts to develop highly sensitive immunoassays has been less successful.

1. HPLC Analysis: The development of HPLC methods addressed two objectives. First, to provide a method for separating radiolabeled glycyl-L-glutamine from glycine and glutamine. This is essential for receptor binding studies with ^{3}H-glycyl-L-glutamine, for which a necessary criterion is to demonstrate that the radioactivity bound to membrane receptors is ^{3}H-glycyl-L-glutamine and not ^{3}H-glycine generated by enzymatic hydrolysis of the radioligand. It may also be necessary to purify ^{3}H-glycyl-L-glutamine to be used for this purpose. A second objective is to develop a method for measuring tissue glycyl-L-glutamine concentrations.

a) HPLC Separation of Glycyl-L-glutamine, Glycine and Glutamine: To develop a method for separating radiolabeled glycyl-L-glutamine from glycine and glutamine, we initially used reverse phase HPLC, a standard method for isolating neuropeptides. We found, however, that glycyl-L-glutamine was poorly retained by reverse phase columns, presumably due to its weak hydrophobic properties. Conversely, we found that glycyl-L-glutamine was not sufficiently polar to be retained on standard anion or cation exchange resins. We therefore tested columns designed to analyze amino acids and found that glycyl-L-glutamine could be completely separated from glycine and glutamine using a Beckman Spherosil A column eluted with 10 mM ammonium acetate containing 50 mM sodium chloride with retention times of 56, 22 and 12 min, respectively. This analytical HPLC method has enabled us to initiate receptor binding experiments because it provides the capability of monitoring the purity of radiolabeled glycyl-L-glutamine.

b) HPLC analysis with NDA derivatization: Subsequently, we developed an HPLC assay, using fluorescent derivatization, initially using ortho-phthalaldehyde (OPA) as a derivatizing agent and separating the glycyl-L-glutamine/OPA derivative by reversed phase HPLC, a standard approach. This was marginally successful but its sensitivity (10 pmol) and resolving power were limited. To overcome these problems, we coupled glycyl-L-glutamine to naphthalene-2,3-dicarboxaldehyde (NDA), a newly developed, more sensitive fluorescent derivatizing agent (Lunte and Wong, 1989) and used a modified RP-HPLC column (VyDac 201HS54). This method substantially improved the sensitivity, linearity (100 fmol to 100 pmol) and resolving capability of the assay. With it, we have been able to detect endogenous glycyl-L-glutamine concentrations in acid extracts of the intermediate lobe of the pituitary. The assay sensitivity is too low to measure brain glycyl-L-glutamine concentrations, although ongoing experiments seek to develop a tissue extraction method which will sufficiently improve its sensitivity. The assay, in its present form is clearly adequate for testing whether glycyl-L-glutamine undergoes metabolism when incubated with immune cells or brain homogenates in lymphocyte proliferation and radioreceptor binding assays, a critically important control. Hence, these experiments succeeded in establishing a relatively sensitive and rapid method for measuring glycyl-L-glutamine concentrations in pituitary extracts and in vitro incubation medium.

2. Glycyl-L-glutamine Assay: Efforts to develop analytical methods for measuring endogenous glycyl-L-glutamine concentrations have been unexpectedly problematic and, despite extensive time investment, only partially successful. Three alternative methods of analysis have been evaluated thus far: RIA, ELISA and HPLC with fluorescent detection. We first attempted to develop an RIA for glycyl-L-glutamine using an antisera generated by Plishka et al. (1985). We found that, like many antisera raised against small molecules coupled to larger proteins (Buijs et al., 1989) the antiserum did not recognize unconjugated [^{125}I]Bolton-Hunter- or ^{3}H-glycyl-L-glutamine and, hence, RIA analysis was not feasible.

We then attempted to establish an ELISA assay by coupling glycyl-L-glutamine to bovine serum albumin (BSA), using either carbodiimide or glutaraldehyde as coupling agents. We found that the antiserum recognized the glycyl-L-glutamine-BSA conjugate by SDS PAGE and Western blotting or by simply applying the conjugate to nitrocellulose filters. Dilution studies indicated that glycyl-L-glutamine immunoreactivity was concentration dependent with respect to both antigen and antiserum. The method was specific for glycyl-L-glutamine to the extent that the antiserum did not recognize closely related dipeptides or ß-endorphin conjugated to BSA. Consistent with our RIA experience, the antiserum did not recognize free glycyl-L-glutamine applied to the nitrocellulose filter, confirming that protein coupling is required for the antiserum to recognize glycyl-L-glutamine.

Further development of a useful ELISA assay proved to be problematic, however. In immunohistochemical studies we had found

that glycyl-L-glutamine displaced antisera labeling of pituitary tissue in which, presumably, glycyl-L-glutamine is conjugated to tissue proteins by glutaraldehyde fixation. We therefore reasoned that, as in other ELISA assays, unconjugated glycyl-L-glutamine should displace antibody binding to the glycyl-L-glutamine-BSA conjugate. This turned out to be the case, but unfortunately, the assay sensitivity was in the micromole range, too low to measure endogenous glycyl-L-glutamine levels. Efforts to increase the sensitivity of the assay were unsuccessful.

3. Immunohistochemical Studies: Immunohistochemical studies designed to map the distribution of glycyl-L-glutamine in the pituitary and brain have also been only partially successful. We were highly successful in labeling intermediate lobe melanotrophs which, from studies of ß-endorphin processing, are known to contain glycyl-L-glutamine (O'Donohue and Dorsa, 1982; Plishka et al., 1985). As expected, anterior lobe corticotrophs, which do not further process ß-endorphin, were not immunoreactive. Immunohistochemical analysis glycyl-L-glutamine's distribution in b revealed immunoreactive axons in the supraoptic, and ot hypothalamic nuclei but the staining intensity was too low to be completely convincing and did not label glycyl-L-glutamine immunoreactive processes in other brain regions. We further evaluated several alternative methodologies but none has substantially improved the staining intensity. These studies were, nonetheless valuable, because no neuronal processes were identified in regions other than those known to be innervated by ß-endorphin releasing neurons, consistent with our hypothesis that glycyl-L-glutamine is synthesized only in POMC neurons. However, further studies will require developing additional antisera. This remains a longer term objective although other avenues of investigation currently hold a higher priority status.

D. Receptor Binding Studies:

We have recently initiated experiments to determine whether saturable and stereospecific glycyl-L-glutamine binding sites are present in brain membrane preparations. To accomplish this objective, we used a standard homogenate receptor binding method (Bennett and Yamamura, 1985) using ^{3}H-glycyl-L-glutamine (5 Ci/mmol). Non-specific binding was determined with 10 μM unlabeled glycyl-L-glutamine. Initially, we used a centrifugation method for separating free and bound ^{3}H-glycyl-L-glutamine, rather than filtration to facilitate testing whether ^{3}H-glycyl-L-glutamine was hydrolyzed during incubation.

In preliminary studies, we found that ^{3}H-glycyl-L-glutamine specifically bound to rat brain membrane preparations. Specific binding was approximately 60% of total binding and was linear over a broad range of protein concentrations. Initial saturation analysis indicated that the binding Kd for ^{3}H-glycyl-L-glutamine binding is 20 nM. HPLC analysis indicates that ^{3}H-glycyl-L-glutamine binding is attributable to the intact dipeptide, rather

than hydrolysis to glycine and glutamine. Glycine displaced ^{3}H-glycyl-L-glutamine binding at very high concentrations; the IC_{50} was approximately 1 mM. These results, while yet preliminary, indicate that specific ^{3}H-glycyl-L-glutamine binding sites are present in rat brain although additional characterization is necessary to determine whether ^{3}H-glycyl-L-glutamine binds to a unique glycyl-L-glutamine binding site or to a previously identified receptor.

SUMMARY AND CONCLUSIONS

A. Pharmacology: CNS Effects

A principal objective of this research, was to determine whether glycyl-L-glutamine is centrally active and if its actions in brain are consistent with its biosynthesis with other POMC peptides. The results demonstrate that glycyl-L-glutamine modulates the cardiorespiratory effects of ß-endorphin but produces no consistent effects on ß-endorphin-induced antinociception suggesting that glycyl-L-glutamine's effects are functionally, and perhaps, anatomically specific. Glycyl-L-glutamine also inhibited α-MSH-elicited hyperthermia, indicating that its modulatory action is not specific to ß-endorphin from which it is synthesized. Glycyl-L-glutamine did not influence these physiological parameters when administered alone, however, suggesting that it functions as an inhibitory modulator but does not act independently. These data provide firm evidence that glycyl-L-glutamine is centrally active.

A second objective, was to explore the possibility that glycyl-L-glutamine's central effects might be replicated by peripherally active analogs. We identified one lead compound, cyclo-glycyl-L-glutamine, which is essentially equipotent to glycyl-L-glutamine when centrally injected and which reproduces the effects of the linear peptide when administered intra-arterially. These data suggest that cyclo-glycyl-L-glutamine permeates the blood-brain barrier by virtue of its lipid solubility, as shown for other cyclic dipeptides using direct assays for blood-brain barrier transport (Hoffman et al., 1977).

In the longer term, the specific effects produced by glycyl-L-glutamine and cyclo-glycyl-L-glutamine may also be of interest from a therapeutic standpoint. Respiratory depression, for example, continues to be a limiting side effect of certain types of therapy with opiate drugs. Although respiratory depression can be controlled with opioid receptor antagonists, such as naloxone, antagonists also inhibit morphine's therapeutic effects. In contrast, glycyl-L-glutamine does not act by blocking opioid receptors and does not appear to inhibit opioid induced anti-nociception, raising the possibility that glycyl-L-glutamine analogs may inhibit the side effects, but not the therapeutic actions of opioids. The finding that glycyl-L-glutamine's effects are anatomically and/or functionally specific is not unprecedented; ß-endorphin-1-27, for example, is a potent antagonist of ß-endorphin-induced analgesia (Nicolas and Li, 1985) but acts as an

agonist in tests of cardiovascular function (Hirsch and Millington, 1991). Nevertheless, we have not, as yet, tested whether glycyl-L-glutamine and cyclo-glycyl-L-glutamine influence morphine analgesia and so further study will be necessary before concluding definitively that glycyl-L-glutamine selectively inhibits the deleterious, but not the therapeutic actions of opioids.

Studies on the thermoregulatory effects of glycyl-L-glutamine also led to unanticipated results. Initial experiments showed that glycyl-L-glutamine inhibits α-MSH-induced hyperthermia when the two peptides are injected into highly specific sites in the mPOA known to contain thermoregulatory neurons as well as relatively high concentrations of POMC-derived peptides. PGE_2 injection into these identical sites also elicits thermogenesis although PGE_2 does not mediate the effects of α-MSH (personal communication, Dr. G. Resch). These observations ultimately led to the finding that glycyl-L-glutamine also blocked the fever induced by PGE_2 and IL-1ß injection into the mPOA. These findings suggest that the function of glycyl-L-glutamine is not limited to the modulation of other co-released POMC peptides. Nevertheless, glycyl-L-glutamine was inactive when injected alone, and thus appears to oppose pyrogenic increases in body temperature without changing normal temperature homeostasis. Hence, glycyl-L-glutamine appears to inhibit perturbations in both cardiovascular and thermoregulatory function without altering the homeostatic regulation of either blood pressure or body temperature when given alone.

B. Pharmacology: Peripheral Effects

Glycyl-L-glutamine also produces trophic and neuroimmune effects in peripheral tissues. We found, in some of the first experiments of the project, that glycyl-L-glutamine induces the expression of the A_{12} form of AChE in neonatal rat ventricular myocyte cultures (Nyquist-Battie, 1993), suggesting the possibility that the dipeptide may be involved in the normal maintenance of AChE expression. This observation was predicated on similar results in skeletal muscle (Lotwick et al., 1990) and sympathetic ganglia (Koelle et al., 1988); together, these finding suggest that glycyl-L-glutamine's trophic action on AChE expression is widespread and not limited to cardiac tissue.

This observation prompted us to consider whether glycyl-L-glutamine may be released locally within the heart and serve an autocrine or paracrine function. Previous studies had localized ß-endorphin immunoreactivity in the heart (Forman et al., 1989) but whether POMC-peptides are actually synthesized by cardiac myocytes was unknown. Moreover, ß-endorphin processing had not been characterized in heart tissue and it was uncertain whether ß-endorphin was converted to ß-endorphin-1-27 and glycyl-L-glutamine. We found that ß-endorphin is almost entirely converted to C-terminally shortened forms, providing strong evidence that glycyl-L-glutamine is also a major product of ß-endorphin processing in the heart. Moreover, *in situ* hybridization and immunohistochemical studies

provided preliminary evidence that POMC gene is expressed within cardiac myocytes. Myocytes also synthesize and release pro-enkephalin-derived peptides (Springhorn and Claycomb, 1989). Thus, the extensive processing of ß-endorphin to non-opioid forms may function as an opioid inactivating mechanism, promoting the action of α-MSH or other POMC-derived peptides. Ongoing studies will investigate the regulation of POMC mRNA in cardiac myocytes.

C. Analytical Studies

A third objective of this research - to map the distribution of glycyl-L-glutamine in brain using both immunohistochemical and quantitative analyses - proved to be more problematic than anticipated and delayed our progress. Glycyl-L-glutamine proved difficult to analyze chromatographically, because it is not sufficiently hydrophobic to be retained by hydrophobic resins, such as reverse phase HPLC, and is not sufficiently charged to be retained by standard ion exchange chromatography. Ultimately, we used a weak cation exchange amino acid column to separate glycyl-L-glutamine from its constituent amino acids and developed a quantitative HPLC method by using NDA derivatization and fluorescent detection with sufficient sensitivity to detect pituitary glycyl-L-glutamine.

These analytical techniques are essential for characterizing glycyl-L-glutamine receptors using ^{3}H-glycyl-L-glutamine. We have now demonstrated that ^{3}H-glycyl-L-glutamine binds to synaptic membrane preparations prepared from rat brain and that binding is displaced by unlabeled glycyl-L-glutamine, linear with respect to protein concentrations and saturable with a Kd of approximately 20 nM. An important prerequisite for these experiments, is the ability to monitor the purity of the ^{3}H-glycyl-L-glutamine and to confirm that binding is wholly attributable to ^{3}H-glycyl-L-glutamine, and not to radiolabeled glycine formed by enzymatic hydrolysis. Experiments now in progress fully characterize ^{3}H-glycyl-L-glutamine binding.

PUBLICATIONS

Fifteen manuscripts were published during the project period, several of which were represent work initiated during a prior USAMRDC funding (86PP6813). In addition, three manuscripts are currently in preparation and we anticipate that at least two other papers well be generated from research initiated under this grant.

A. Journal Articles:

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5. Lavigne, G.L., Millington, W.R. and Mueller, G.P. The CCK-A and CCK-B receptor antagonists, devazepide and L-365,260, enhance morphine antinociception only in non-acclimated rats exposed to a novel environment. Neuropeptides 21:119-129, 1992.

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13. Chronwall, B.M., Dickerson, D.S., Huerter, B.S., Sibley, D.R. and Millington, W.R. Regulation of heterogeneity in D_2 dopamine receptor gene expression among individual melanotropes in the rat pituitary intermediate lobe. Mol. Cell Neurobiol. 5:35-45, 1994.

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15. Unal, C.B., Owen-Kummer, M.D. and Millington, W.R. ß-Endorphin-induced cardiorespiratory depression is inhibited by glycyl-L-glutamine, a dipeptide derived from ß-endorphin processing. J. Pharmacol. Exp. Ther. (Submitted).

B. Publications In Preparation:

16. Resch, G.E. and Millington, W.R. Interleukin 1ß and PGE_2 induced fever are blocked by glycyl-L-glutamine. (To be submitted to Brain Research).

17. Unal, C.B., Owen-Kummer, M.D. and Millington, W.R. Inhibition of morphine induced hypotension and respiratory depression by glycyl-L-glutamine and cyclo-glycyl-L-glutamine. (To be submitted to J. Pharmacol. Exp. Ther.).

18. Millington, W.R. and Manning, A.B. Opioid peptides: Analytical methods for the simultaneous measurement of multiple peptides derived from common prohormones. (To be submitted to Methods in Toxicology).

C. Abstracts:

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4. Lavigne, G.J. and Millington, W.R. The CCK-A and -B antagonists, devazepide and L-365,260, potentiate morphine antinociception, but only in non-acclimated rats. Third IBRO World Congress of Neuroscience, 1991.

5. Dickerson, D.S., Pratt, B.S., Millington, W.R. and Chronwall, B.M. D_2 dopamine receptor regulation in the intermediate lobe of the rat pituitary. Society for Neuroscience, 1991.

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10. Bernard, L.H., Chronwall, B.M., Evans, V.R. and Millington, W.R. Post-translational processing of ß-endorphin and ACTH in the human pituitary. American Society for Anesthesiology, 1991.

11. McQuillan, R., Lavigne, G.L., Fibuch, E. and Millington, W.R. ß-Endorphin-induced antinociception is modulated by products of its post-translational processing. Midwest Anesthesiology Residents Conference, 1992.

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15. Owen-Kummer, M.D., Unal, C.B., Fibuch, E.E. and Millington, W.R. Glycyl-L-glutamine (ß-endorphin-30-31) inhibits the central cardioregulatory effects of ß-endorphin-1-31. Midwest Anesthesiology Residents Conference, 1993.

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21. Pendergrass, D. and Millington, W.R. ß-Endorphin inhibition of T-lymphocyte proliferation is attenuated by glycyl-L-glutamine. Missouri Academy of Sciences, 1994.

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